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(54) Title: SEPARATION OF X- AND Y-SPERM CELLS

(57) Abstract: The present invention relates to separation of X- and Y-sperm cells from each other by centrifugation in a colloidal density medium. In the method X and Y sperm cells can be separated from each other in a medium formula which has a density close to that of X and Y sperm cells of the species in question. The method is simple and rapid and provides highly purified X and Y sperm cells, respectively in high yield.

SEPARATION OF X- AND Y-SPERM CELLS

Field of the Invention

The present invention relates to separation of X- and Y-sperm cells from each other by centrifugation in a density gradient medium.

Background of the Invention

There has long been a large interest within specially animal breeding and production to be able to separate X- and Y-sperm cells from each other and to thereby have the ability to choose sex of the progeny, for example for bovine animals only cows for milk production and only bulls for meat production. Many different techniques have been tried but there is no single technique that fulfils all commercial demands.

The techniques that have claimed to be successful in a qualitative manner are immuno-magnetic separation and flow cytometry which report an enrichment of X- and Y-sperm cells, respectively, of up to 98-99% (A.T. Peter, P.P. Jones and J.P. Robinson, Theriogenolgy 11 -1184, 1993; Sex pre-selection by DNA: Uptake on success of flow cytometric sperm sorting for shifting the sex ratio to 90:10 or more. L.A. Johnson, J.R. Dobrinsky and G.R. Welch. Abstract P24-1, The 13th International Congress On Animal Reproduction, Sidney, Australia 1996). These techniques are technically very complicated and give too few sperm cells per time unit to make them suitable for routine use.

Experiments have been made with Percoll™, a colloidal density medium, to separate X- and Y-sperm cells from each other by discontinuous and continuous density centrifugation but the results have not been promising; only a 60-70% enrichment with discontinuous technique and no enrichment at all with the continuous technique has been reported (About influence of bull on selective enrichment of X-and Y-spermatozoa by Percoll™ density

gradient centrifugation. W. Lange, H. Pemsell, S.Blottner, R. Roselius, J. Pfeilsticker and P. Rommel, Arch. Tierz., 38, 2, 156-161, 1995; Attempted sexing of bovine spermatozoa by fractionation on a Percoll™ density gradient G.C. Upreti, P.C. Riches, and L.A. Johnson, Gamete Research 20:83-92, 1988).

In U.S. 4,927,749 a colloidal density gradient medium is described which is said to enable separation between X- and Y-sperm cells (Example 11) by continuous density centrifugation in said medium for10 - 15 minutes at 2.000 x g and a start density of 1.185 g/mL, which is said to be between the densities of human X and Y sperms. Any recoveries or enrichment numbers are not presented in the patent and there are no verifying scientific reports, and considering the parameters given therein it is seriously questioned whether this method has actually been verified. Furthermore the sperm cells are not separated from debris and other possible contaminants with this technique. Accordingly, the sample is subject to a separation that most probably is impaired be the polydisperse nature thereof. More importantly, sperm cells separated according to USP 4,927,749 will be of a very low viability and the product is therefore not useful for breeding purposes.

Summary of the Invention

The present invention provides a simple and rapid method of separating X and Y sperm cells from each other in high yield and with high purity.

The present invention relates to a method to separate X- and Y-sperm cells from each other in a semen sample in a density gradient medium by centrifugation. The method comprises the following steps;

a) Providing a sample comprising X and Y sperm cells;
b) Removing any other undesired components than X and Y cells from the sample to provide a purified sample without contaminants;
c) placing

i) a density gradient medium being selected to have a density close to that of X- and Y-sperm cells; and

ii) the semen sample

in a centrifuge tube;

d) centrifuging said tube to achieve separation of X and Y sperm cells from each other in said medium; and

e) collecting X and Y sperm cells separately.

Above, it is to be understood that during step (b), the components of the sample removed can be any undesired contaminants present in sperm plasma or an ejaculate, including incompetent sperm cells, sperm heads, cell debris, leukocytes, micro-organisms etc. Accordingly, defect or abnormal cells, that are not useful for fertilisation purposes, are removed in step (b). The sample is often provided in saline. As the skilled in this field will realise, for best results, the present method is run at a temperature where the X and Y sperms are inactive i.e. where they do not themselves create any movement that disturbs the separation above. Thus, the method is run at a temperature where the X and Y cells behave as much as particles as possible, such as below room temperature, such as below 15^{0}C or preferably below about 6^{0}C.

Without wishing to be bound by a specific theory, it appears probable that the superior separation results obtained by the present invention are due to a combination of sedimentation, flotation and density. The present method can be viewed as a three parameter system comprising size, density and exclusion of sperm cells in a colloidal medium, e.g. of 15-30 nm particles, or in a specific case below that range, such as about 2-3 nm particles, concentrated to 45% by weight of silica, which as the skilled in this wield will understand is not comparable with the above discussed USP 4,927,749.

In one embodiment, the present centrifugation is isopycnic meaning that it is done to equilibrium so that each kind of cell locates at its true buoyant density in the medium.

Alternatively, the centrifugation is stopped before equilibrium is reached (rate zonal conditions) which means that a difference in density and/or size between the cells will affect the separation pattern obtained. In this case, the separation is run until a satisfactory separation is obtained. To decide a suitable time for a specific system, a number of test centrifugations are easily run, to enable the drawing of a calibration curve.
In a first case of rate zonal centrifugation based on density, densities of the medium formula are selected at values close to that of the sperm and more specifically at a lower value if a sedimentation is desired and at a higher value if a flotation is desired.
In the alternative rate zonal centrifugation, the density of the medium formula is set at relatively different value than that of the sperm, the farther away the better result. For the different centrifugation techniques reference is made to the second edition, revision 2 of Percoll™ Methodology and Applications, Amersham Biosciences AB. The velocity of the sperm cells movement in the tube can be described by the following equation:

$$V = dr/dt = [D^2(\rho_s - \rho_m)\, g] \,/\, 18\eta$$

wherein

D is the diameter of the cell
ρ_s is the density of the sperm
ρ_m is the density of the medium and
η is the viscosity of the medium used.

The sample may be placed on top of said medium or beneath it. Optionally, said medium in step c) is treated to form a density gradient before said

sample is added to said centrifuge tube. Such a preformed gradient can for example be formed by centrifugation or mixing. The gradient is preferably very flat or essentially planar, i.e. it comprises a very small density difference in the centrifuge tube, at least at the centre of the gradient.

The gradient may be in different forms such as discontinuous, continuous or is represented by a constant density. If the gradient is continuous, it may be steep in the ends and more or less planar in the central portions. It is important that the gradient provides the necessary separation which means that it should be possible to recover fractions where X and Y sperms, respectively, are enriched to at least 70% or more, preferably 100%.

As mentioned above, the semen sample is purified by discontinuous centrifugation in step b) before separation.

An alternative way is to purify the sample by sedimentation and flotation described in our co-pending SE 00 04271-3. The centrifugation medium used according to the invention should be a heavy medium, such as a colloidal silica-based material. The medium should be inert, autoclavable in the presence of salt, have a low or no endotoxin content, an as low osmotic pressure as possible, preferably below 20 mOsm/kg, a low viscosity in salt (< 5cP), at high density (>1.3 g/ml; RG). One representative example of a suitable medium is ReadiGrad™ (in general denoted RG and available from Amersham Biosciences, Uppsala, Sweden). Alternatively, silica particles can be bought from commercial sources, such as Nyacol, and silanised according to well known techniques by the user. Such a medium can then be prepared into a medium formula in order to set the desired pH and osmolality for each specific embodiment. Below, a medium prepared in such a way but based on the above mentioned ReadiGrad™ will be denoted an RG formula. In an illustrative embodiment, an RG formula useful in the present

method exhibits a pH of about 6-8, such as about 7, and an osmolality of bout 200-400, such as above about 300, preferably about 350.

As compared to USP 4,927,749 discussed above, wherein a 27% silica solution is used (particles of 7-220 nm), the nature of the above described medium will provide a very different viscosity effect.

In one embodiment, the present method utilises a semen sample derived from a non-human mammal. When the sample is bovine, the density referred to above is close to that of the X and Y sperm cells, i.e. about 1.120g/mL. The corresponding values for human cells are substantially the same. In general, depending e.g. of the source of the sample and the separation principle used (isopycnic or rate zonal conditions), the present invention utilises a density range of 1.05-1.30, such as 1.06-1.13, and especially about 1.12 g/ml. These densities are applicable under the experimental conditions mentioned below in the experimental part. The present invention does not relate to centrifugations during which human X- and Y-sperm cells, respectively, have densities centred around 1.185 g/mL.

In the above method, the separation may be improved by manipulating the density of X and Y sperm cells, for instance by altering pH, osmolality of the sample and/or medium. The manipulation may comprise swelling and/or shrinking of X- and Y-sperm cells.

In an alternative way, the manipulation may be achieved by derivatising of X and Y with different chemical and/or biological compounds.

All the above mentioned centrifugations for separation of X and Y cells from each other in a density gradient as defined above will be performed either under isopycnic conditions or under rate zonal conditions depending on which kind of difference (buoyant density or size) the separation shall focus at.

The invention also relates to a method for separation as above, wherein said sample is mixed with said medium to achieve a density of said mixed sample-medium which lies close to the density of X and Y sperm cells. In this case, the separation is mainly achieved by isopycnic centrifugation. The separation pattern obtained in this variant of the invention will thus preferentially be based on the different buoyant densities of the sperm cells.

The density medium may be any colloidal density medium suitable of forming the above mentioned types of gradient for X and Y sperm separation. A preferred medium is a colloidal density medium. An example of a colloidal density medium is the ReadiGrad™ (RG) formula described in our co-pending SE 00 04271-3, which is referred to and incorporated herein by reference.

EXPERIMENTAL PART

General description of an exemplifying embodiment

1. The aqueous ejaculate, mixed with RG formula (= the colloidal medium described above to a density of 1.057 g/mL, is placed in the bottom of the centrifuge tube, overlaid with RG formula of a density of 1.030 g/mL and on top a solution of 0.150 M NaCl and this is then centrifuged about 15 minutes at 10.000 x g_{av}. In this step lighter particles are flotated. Sperms and the remaining contents of the ejaculate are found in the lower phase.

2. The resulting lower phase resuspended and mixed with RG formula to a density of 1.200 g/mL is placed in the bottom of a new centrifuge tube, overlaid with RG formula of a density of 1.090 g/mL and on top of this solution of 0.150 M NaCl and then the tube is centrifuged for about 15 minutes at 1.000 x g_{av}. Deformed and immature sperms, cells (usually leukocytes) and possible protozoans from the ejaculate are present in the

upper phase layer. Sperms, sperm heads and the remaining content in the ejaculate are present in the lower phase.

3. The resulting lower phase is resuspended and transferred to a new centrifuge tube, overlaid with RG formula of a density of 1.130 g/mL and on top of this solution of 0.150 M NaCl and then the tube is centrifuged for about 15 minutes at 1.000 x g_{av}.
Intact, viable sperms from the sample are now present in the upper phase layer while bacteria, virus, sperm heads and the remaining contents of the ejaculate are present in the lower phase.

4. The thus separated, concentrated and purified sperms are now
(a) mixed with RG formula to a density corresponding to the mean density of natural mixture of X and Y sperm cells, typically 1.120 g/mL,
(b) placed in a new centrifuge tube, and
(c) centrifuged until separation is accomplished, i.e. to the appearance of one band enriched in x-sperms and another band enriched in y-sperms.
In a typical case separation is accomplished after about 1 hour, depending on rotor type (angle, swing-out or vertical rotor) at 1.000 x g_{av}. In the preferred case, the swing-out rotor is used.

By the three first steps the intact and viable sperms are separated from functionally incompetent sperms, sperm heads and other rests of sperms, cells and cell debris, micro organisms and also from everything else in the ejaculate including dissolved substances and free radicals which otherwise can accompany the sperms and have a negative influence on for example a later insemination. Accordingly, a purified sample is obtained. In this context, it is to be understood that the term "purified" means that any components of the original semen sample that could harm the separation of X and Y sperm cells have been removed or essentially removed to an extent where a satisfactory separation can be achieved.

In the last step X- and Y-sperm cells are separated from each other on basis of their behaviour during the centrifugation. Before the system reaches equilibrium (rate zonal conditions) the difference in size affects the separation. After some time the system will reach equilibrium and the different cells will be located at the density corresponding to their buoyant density. The sizes of the cells is likely to have an small or insignificant effect on the separation pattern obtained under this latter condition. Typically the difference in buoyant density of X and Y sperm cell is very small, for instance in the magnitude of 0.0010 g/mL. The difference can alternatively be expressed in percentages, in which case the desired difference is < 1%. This means that the centrifugation conditions must be selected accordingly, i.e.

(a) isopycnic conditions with development of a gradient that is flat enough for separation at the same time as the time and g-force enable that all sperms are allowed to sediment or flotate to their respective densities, or

(b) rate zonal centrifugation so that the difference in size will assist in the separation.

The physical separation between X- and Y-sperm cells then becomes large enough (e.g. several 1, 2 , 3 or more such as up to several cm) so that the sufficient, such as almost 100%, enrichment of X- and Y-sperm cells, respectively, is maintained during the following gradient fractionation.

Because the sperms have been concentrated after step three and the illustrative RG medium formula has a density of 1.40 g/mL which enables a dilution of about 1:2.3 with the concentrated sperms before step four, large volumes of ejaculate can be processed per time unit. Furthermore, the technique is simple to perform which makes the method cost effective and well suited for routine use.

In the following, two alternative Examples are provided to illustrate but not to limit the invention.

Example 1 Purification of X- and Y-sperms

Pooled ejaculate from three healthy bulls were mixed, after dilution 1:10 with 0.15 M NaCl, with RG formula to a final density of 1.057 g/ml in 8 different 50 mL tubes (17 mL 1:10 ejaculate + 2.8 mL RG formula) The mixtures are transferred to new centrifuge tubes (50 mL), overlaid with 5.0 mL of a RG formula of a density of 1.030 g/mL and on top of this 5.0 mL of a 0.15 M NaCl solution. Centrifugation was then performed in a swing-out rotor at 10.000 x g_{av} for about 15 minutes. The upper phase was aspirated with a Pasteur pipette and discarded. The lower phases (about 20 mL) were resuspended, transferred to new tubes and mixed with 15 mL RG formula to obtain a density of 1.200 g/mL, overlaid with 5.0 mL of a RG formula of a density of 1.090 /mL and on top of this 5.0 mL of a 0.15 M NaCl solution and then this was centrifuged at 1000 x g_{av} for about 15 minutes. The upper phases were then aspirated with a Pasteur pipette and discarded and then the lower phases (approx. 20 mL) were resuspended, transferred to a new tube, overlaid with 5.0 mL RG formula of a density of 1.130 g/mL and 5.0 mL of a 0.15 M NaCl solution and then centrifugation was performed at 1000 x g_{av} for 15 minutes. The tubes were fractionated from above with an automatic pipette in 0.9 mL fractions. Fraction 6 to 10 from the eight fractionated centrifuge tubes containing the purified sperms were pooled (approx. 3.6 mL).

Following the pre-purification steps above, the purified X and Y cells are separated from each other according to the invention according to one of the following principles:

a) Isopycnic conditions, i.e. according to buoyant densities; or
b) Rate zonal conditions, i.e. the sizes of the different cells affect the separation pattern.

In both alternatives, a density gradient medium is used having a density close to the density of X and Y cells from the species in question.

In a) the sample may be mixed with RG formula or overlaid on RG formula. In b) the sample is overlaid on RG formula.

Example 2 Purification and separation of X- and Y-sperms from bull

Fresh semen from a normal bull was layered after precooling (+6°C) on a discontinuous gradient comprising 20 mL 80% formulated RG (10mM HEPES, pH=6.9, density=1.10g/mL, osmolality=350 mOsm/kg) and 10 mL 40% formulated RG(10mM HEPES, pH=6.9, density=1.05g/mL, osmolality=350 mOsm/kg). Following centrifugation in a swing-out rotor at about 800 g_{av}, +6°C, for 30 minutes the purified sperms were harvested from the bottom part of the 80% layer. To separate X- and Y-sperms from each other, aliquots of 0.5 mL(about $0.5x10^9$ sperms) of the purified sperms was mixed either with a)10.650 +/- 0.0005 g of formulated RG (10mM HEPES, pH=6.9, density = 1.1210 g/mL, osmolality=350 mOsm/kg) or with b) 10.800 +/- 0.0005 g of formulated RG (10mM HEPES, pH=6.9, density = 1.1368 g/mL, osmolality=350 mOsm/kg) and the mixed samples were centrifuged in a swing-out rotor at about 3.000 g_{av}, +6°C, for 60 minutes. Following centrifugation fractions enriched in Y-sperms were recovered from the top of the gradients in a) and fractions enriched in X-sperms were recovered from the bottom fractions in b).

CLAIMS

1. A method to separate X and Y sperm cells from each other in a semen sample in a density gradient medium by centrifugation, comprising the following steps

(a) Providing a sample comprising X and Y sperm cells;
(b) Removing any other components than X and Y cells from the sample to provide a purified sample without contaminants;
(c) placing
i) a density medium selected to have a density close to the that of X and Y sperm cells ; and
ii) the sample obtained from step b)
in a centrifuge tube;

(d) centrifuging said tube to achieve separation of X and Y sperm cells from each other in said medium; and
(e) collecting X and Y sperm cells separately.

2. A method according to claim 1, wherein the colloidal density gradient medium is defined by a pH of between about 6 and about 8 and an osmolality of between about 200 and about 400.

3. A method according to claim 1 or 2, wherein in step c), a density gradient is formed in said medium before said sample is added to said centrifuge tube.

4. A method according to any of the above claims, wherein said gradient is discontinuous, continuous or is represented by a constant density.

5. A method according to any of the above claims, wherein said sample is derived from a non-human mammal.

6. A method according to claim 5, wherein said sample is purified during step b) by discontinuous centrifugation.

7. A method according to claim 5, wherein said sample is purified is purified during step b) by sedimentation and/or flotation.

8. A method according to one or more of the above claims, wherein said separation is improved by manipulating the density of X and Y sperm cells.

9. A method according to claim 8, comprising swelling of X and Y.

10. A method according to any of the above claims, wherein said sample is mixed with said medium to achieve a density of said mixed sample-medium which lies close to the density of X and Y sperm cells.

11. A method according to any of the above claims, wherein said density medium is a colloidal density medium.